

## Research Article

# Isolation, biochemical and molecular detection of *Bacillus subtilis* and *Bacillus licheniformis* from the digestive system of rainbow trout (*Oncorhynchus mykiss*) and its inhibitory effect on *Aeromonas hydrophila*

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Received: August 2019

Accepted: June 2020

### Abstract

Our study was focused on characterization of dominant *Bacillus* bacteria isolated from the digestive tract of *Oncorhynchus mykiss*. After 8 weeks, the fish fed with probiotic-free diet were dissected under strictly controlled conditions. Fish intestines were eviscerated and its content diluted to  $10^{-4}$  using Ringer solution. Two isolated bacteria were Gram-positive, catalase-positive, and oxidase-negative, based on the biochemical tests. A mixed of the two strains was the most efficient isolates determined in *O. mykiss*. Molecular characterization using PCR-Ribotyping was carried out using sequencing the 16S rRNA gene of both strains, which isolated two bacteria, *Bacillus subtilis* Ahari.H1 and *B. licheniformis* Ahari. H2. The isolated bacteria could tolerate 0.3% bile salt and non-hemolytic in nature. They also showed resistance to pH at 2.5 and 4 for acid test and 2.3 and 7 for gastric juice, pepsin and trypsin. It is concluded that the efficiency of the isolated probiotics can be candidates as probiotic in fish culture. It was unveiled that these strains can successfully diminish *Aeromonas hydrophila* pathogenicity in rainbow trout.

**Keywords:** Rainbow trout, Probiotic, *Bacillus subtilis* Ahari.H1, *Bacillus licheniformis* Ahari.H2, PCR

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## Introduction

Since global fish food consumption per capita demand is on the rise, the requests for aquaculture and fish production significantly increased (Gabriel *et al.*, 2015). The interest for salmonid farming has been widely extended so that nowadays salmonid industry becomes one of the most major economic activities in Oceania, America, Africa, Asia, and Europe (Engle *et al.*, 2019). Iran is a top-ranked rainbow trout culture country throughout the world with 173384 MT production in 2018 (IFO, 2019). This increase leading to a growth in fish stock density, which subsequently results in different lethal viral and bacterial outbreaks and huge economic losses. This sound for thoughtful management of outbreaks of diseases and deployment of restrict biosecurity measures and application antibiotic alternatives to prevent diseases. *Bacillus* bacteria have been investigated extensively as presumed probiotics to enhance immune system of fishes due to some unique attributes (Zhang *et al.*, 2011). Numerous strains of *Bacillus* represent uncommon biological appearances such as spore-forming feature enabling them to resist to destructive environmental conditions of digestive tracts of fish and shrimps, which are suggested to be applied as probiotics to fish feed to remediate intestine microbial fauna to dominate to pathogenic bacteria (Soltani *et al.*, 2019).

In order to bioremediation of aquaculture rearing water and intestine microbial community, Application of *Bacillus* spp. as probiotics to feed or water, has been extended among fish farmers. Isolation and Identification of bacteria gained from digestive tract of fishes has been well investigated in last century. Some of the bacteria have probiotic potent, which can increase immunity in fish and positively change bacterial community in gut (Hoseinifar *et al.*, 2018).

Fifty strains isolated from the intestines of *Sparus aurata* were examined and proved the protecting characteristics of *Vagococcus fluvialis* experimented in the European bass (*Dicentrarchus labrax*) against *Vibrio anguillarum* (Sorroza *et al.*, 2012). Similarly, Characteristics of lactic acid bacteria (LAB) isolated from the rainbow trout showed their inhibitory activities against *V. salmoninarum* and *Lactococcus garvieae* in the same fish species (Didinen *et al.*, 2018). Likewise, the characteristics of *Leuconostoc mesenteroides*, a new probiotic isolated and evaluated from the intestine of *Channa striatus* was identified (Allameh *et al.*, 2012). Some tests such as oxidase can be used to identify the suspected bacteria whether it could be the coliform bacteria (all negative Gram) or belonging to other genera such as *Aeromonas*, *Pseudomonas*, *Neisseria*, *Campylobacter*, and *Pasteurella* (positive Gram) (Nedelkova *et al.*, 2019).

Some researchers showed if *B. subtilis* or *B. licheniformis* are added to feed of *O. mykiss* the value of survival rate can be increased while they exposed against *Yersinia ruckeri* (Raida *et al.*, 2003) and *Aeromonas* sp. (Newaj-Fyzul *et al.*, 2007) of which also showed stimulation of immune parameters, particularly respiratory burst and gut lysozyme. The effect of the two probiotics, *B. subtilis* and *B. licheniformis* on some criteria of *Oreochromis niloticus* showed that weight gain, specific growth rate, and feed conversion ration as well as lysozyme, superoxide dismutase activities, and immunoglobulin M level were highest in the treatment of 10 g kg<sup>-1</sup> of the probiotic (Abarike *et al.*, 2018).

With this background in mind, the aim of this study was the isolation and molecular detection of *predominant Bacillus* strains from the digestive system of *O. mykiss* based on the 16S rRNA gene and assessment the probiotic potential test on the isolated bacteria.

## Materials and methods

### *Preparation and transfer of fish*

A total of 80 healthy juvenile fish of *O. mykiss* with an average weight of 10 g and 80 juvenile fish with an average weight of 25 g were randomly selected from a farm in Firoozkooh, north of Iran, and transferred to the farm, provided in National Institute of Genetics and Biotechnology of the Ministry of Science, Research, and Technology. The fish were distributed

among twelve fiberglass tanks (1000 L) disinfected with betadine 10%, and rinsed with water, well aerated with an air blower (2 hp; Hila-China). They were not fed for 48 hours in order to acclimate them to the new environmental conditions, including temperature, oxygen, and pH, and to reduce their stress. After this period, they were given probiotic-, prebiotic-, and inhibitor-free feed (Kimiagaran-e Taghziyeh, Iran) for 8 weeks, 3 times a day.

The water used for breeding was examined in terms of chlorine, pH, dissolved oxygen, ammonia, nitrite and nitrate, salinity, turbidity, total hardness, electrical conductivity, sulfate, total phosphate, total dissolved solids, total suspended solids, total coliform, and total count. After comparison with the standard values, the results showed that the water could be used for fish breeding as optimum (Nafisi Bahabadi, 2010).

### *Preparation of samples*

In order to ensure that the probiotics could be isolated, bacterial samples were designed to be isolated from two groups of fish so that all the fish with an average weight of 25 and 40 g were evacuated from the tubs to containers, sacrificed, and transferred to the laboratory (in ice). All the instruments and fish were disinfected with alcohol at the laboratory. The intestines were removed with a cut under the belly and then weighed. Ringer's solution (9v:1w) was added to the intestinal samples. The

Stomacher<sup>®</sup> device was used to mix and homogenize the samples. After preparing a  $10^{-1}$  dilution, the process was continued to reach  $10^{-4}$  (Chinachoti *et al.*, 2019).

#### *Isolation and Identification of bacterial strains*

The documentation of the dominant bacterial strains was achieved according to morphological, biochemical and molecular attributes.

#### *Morphological characterization*

Morphological aspects including colony features (color, form, edge and surface) and cell attributes (general feature and gram staining) of the isolated bacterial strains were assessed

#### *Sample culture*

**Tryptic Soy Agar:** Tryptic Soy Agar (Merck, Germany) with thermal shock and De Man, Rogosa, and Sharpe (Merck, Germany) were used to cultivate *Bacillus* bacteria and to determine the total count of probiotic bacteria. The homogenized solution and Ringer's solution were added to a bain-marie (Memmert, Germany) at 80°C for 10 minutes until the active bacteria disappeared. The mixture was then immediately cooled down by cold water and placed in an incubator (Memmert, Germany) at 37°C for 45 minutes. It was left to rest for 45 minutes so that spores could germinate in the presence of bacilli. Afterwards, the solution was poured into a plate, and TSA was added; then incubated at 37°C for 72

hours under aerobic conditions (Alonso *et al.*, 2019).

In this study, we intend to identify and cultivate a common series of microorganisms having properties of probiotics. These probiotics were *Pediococcus acidilactici*, *Lactobacillus acidophilus*, *Enterococcus faecium*, *L. casei*, *L. plantarum*, *L. delbrueckii* subsp. *Bulgaricus*.

#### *Biochemical characterization*

The bacterial strain was exposed to different biochemical tests such as antibiotic application, motility, urease, oxidase, catalase, Voges Proskauer (VP), galactose, anaerobic and aerobic growth. Due to the aerobic condition of the bacteria; *Enterococcus* sp. and *B. subtilis*, they were respectively cultured using Bile Asculin Azide agar and TSA but MRS agar medium applied for the rest based on the method of Vandera *et al.* (2018) with minor modification. It is noteworthy that *B. subtilis* and probable *Enterococcus* sp. were aerobic, the plates of the two above bacteria directly incubated at 37°C. Antibiotics including vancomycin, novobiocin and antifungal compound nystatin was used for growth of *Pediococcus* (Lee and Lee, 2009) and sorbitol and reagents of bromocresol (Bujalance *et al.*, 2006) as well as ciprofloxacin (Schillinger and Holzapfel, 2011) were applied to grow *L. plantarum*.

In the case of *Bacillus* sp., the culture medium was used instead of antibiotics, in which the homogenized intestinal solution and Ringer's solution

were placed in a bain - marie boiling at 80°C for 10 minutes to kill the active bacteria. Immediately, the obtained solution was cooled by cold water, then incubated at 37°C for 45 minutes (so-called dormant state) to germinate spores of *B. subtilis*. The final solution was then poured into a plate; the TSA medium was added and incubated at 37°C for 72 hours. Instead of the antibiotic application to grow *L. bulgaricus*, pH alteration (Reyes *et al.*, 2018) was the approach in this study so that the medium temperature was reduced to about 50°C after using glacial acetic acid and the pH reached to 4.5-5.5.

The culture medium of *Enterococcus* sp. was a completely selective one with sculin and bile making the environment to grow only *Enterococcus* sp. As mentioned earlier, the solution inside the Stomacher bag formed our base dilution. Then it was diluted up to 10<sup>-4</sup> using ringer solution and was individually poured into four MRS agar plates to measure the total count of probiotic bacteria and cultured the above-mentioned bacteria specifically. The considered plates were then placed in an anaerobic jar to prepare anaerobic conditions and incubated at 37°C for 72 hours (AlKalbani *et al.*, 2019).

#### *Molecular characterization*

##### *Molecular and sequence analysis*

The PCR based on 16S rRNA gene was applied, and the sequence to detect the isolated bacteria. The molecular detection of the isolated bacteria from

rainbow trout was carried out at the Children's Medical Center of Imam Khomeini Hospital, Tehran, Iran. (Weisburg *et al.*, 1991; Haditomo and Prayitno, 2018).

##### *DNA extraction*

The boiling method was used for DNA extraction. For this purpose, a loop of bacterial colony was collected using a sterile needle and then added to a microtube containing 500 µL of distilled water following by vortexing the solution. The microtubes were then placed in the water wells of a block heater (SBH200D Stuart, UK) for 20 minutes at 95°C and centrifuged for 5 minutes at 13000 rpm (Eppendorf, Germany). The DNA-containing solution (supernatant) was extracted and stored at -20°C until further analysis (Sung *et al.*, 2003; Shahbazi and Narenji, 2014). As shown in the Table 1, the following primers were used for PCR (Weisburg *et al.*, 1991).

##### *Preparation of agarose gel*

Agarose powder 1% and TBE buffer (0.5X) were used to prepare the agarose gel. The buffer was prepared by mixing 54 g of tris base with 27.5 g of boric acid (H<sub>3</sub>BO<sub>3</sub>) and 4.65 g of EDTA. Distilled water was used to reach a volume of 500 mL; it was then diluted using 0.1 X distilled water to prepare the TBE buffer (0.5X).

**Table 1: Primers used in this study.**

Primer name	Nucleotide sequence (5' to 3')	GenBank Accession Number
FD1	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG	MF375213.1
Rp2	CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT	KY681777.1

Subsequently, a portion of the 1% agarose powder was mixed with 40 mL of TBE buffer (0.5X) and boiled using a Hot Plate for clarity and transparency (Mojabi and Mojabi, 2015).

#### *PCR amplification of bacteria*

A 25  $\mu$ L of master mix containing FD1 and Rp2 primers (0.5  $\mu$ L each), 0.5  $\mu$ L 10mM dNTPS Mix, 0.5  $\mu$ L 25mM MgCl<sub>2</sub>, 2.5  $\mu$ L PCR Buffer 17.5  $\mu$ L PCR grade water, 3  $\mu$ L purified DNA (3  $\mu$ L PCR water instead of DNA as negative control) and 0.2 Taq DNA polymerase were gently vortexed and used it in PCR assay (Morimoto *et al.*, 2018).

The PCR assay was performed at a volume of 25  $\mu$ L (Table 3) in a thermal cycler (2720, Applied Biosystems) in 35 cycles. The PCR assay was carried out in the following steps: initial denaturation at 94°C for 2 minutes followed by secondary denaturation at 94 °C for 45 seconds; pattern-binding to primer at 55°C for 45 seconds; extension at 72 °C for 60 seconds; and a final extension at 72°C for 15 minutes. At the end of the PCR, the microtube lids were opened and incubated at 45°C for 45 minutes to increase the final concentration of the products and hold them overnight at 10°C. The PCR products were inserted in the Gel-Doc

system (BioRad), and the bands were observed using a UV lamp (Jasem Saleh, 2014) Finally, the samples were sent to Bioneer (Seoul, Korea) for sequencing.

#### *Sequence analysis*

The synthesis of primers (Table 1) and identification of *B. subtilis* and *B. licheniformis* strain was performed based on 16S rRNA sequencing analysis by Bioneer (Seoul, Korea). Sequencing results of the amplified product was analyzed by Sanger's method using 96 capillaries and a 3730xl-automated sequencer (Applied BioSystems, USA). Forward and reverse sequences were aligned to obtain consensus sequence by BioEdit version 7.0.9.0 (Tom Hall, Ibis Biosciences, Carlsbad, California) based on the method of (Imran *et al.*, 2018) and the similarity search was also performed using the BLASTn and NCBI web services (<http://blast.ncbi.nlm.nih.gov>, December, 2019). The comparison of the conserved motif of 16s rRNA genes were carried out by DNAMAN and GeneDoc version 2.6.002 programs.

#### *Nucleotide sequence accession numbers*

Sequence data were deposited in the DNA Data Bank of Japan (DDBJ),

which is available under the accession numbers MN809400 (*Bacillus subtilis* Ahari.H1) and MN809401 (*B. licheniformis* Ahari.H2).

#### *Differential tests*

After the molecular analyses, the plates were transferred to the laboratory of the Faculty of Veterinary Medicine, Islamic Azad University, Science and Research Branch, Tehran, Iran. First, subcultures were prepared from each plate. After 48-hour incubation at 37°C, hemolytic activity, catalase and oxidase tests, as well as Gram staining and potassium hydroxide (KOH 3%) were performed. Resistance to acid, bile oxalate, Trypsin and pepsin tests were used to identify and confirm the bacteria as probiotics (Barrow and Feltham, 2004; Shabani, 2008 (In Farsi)).

#### *Preparation of microbial suspension*

The detected bacteria were cultured in Tryptic Soy Broth at 37°C for 24 hours. Afterwards, the broth was centrifuged at 5000 g for 10 minutes. The supernatant was collected, and the precipitates were washed with 0.1 M Phosphate-buffered saline at pH 7. The tubes containing the suspension were then compared with the PBS tubes with 0.5 McFarland turbidity (Shabani, 2008; ISIRI, 2014) using spectrophotometer (Eppendorf, Germany), which showed the ultimate concentration of 10<sup>8</sup> cell/mL for both bacteria, which subsequently diluted to 10<sup>6</sup> and 10<sup>4</sup> cell/mL, respectively

produced from dilutions of 10<sup>-4</sup>, 10<sup>-6</sup> and 10<sup>-8</sup>.

#### *Resistance to acid*

The 100 µL of bacterial suspension were then added to 2 bottles prefilled with 10 mL of tryptic soy broth (TSB; Merck) adjusted to pH at 2.5 and 4. Each pH was triplicated for both bacteria, *Bacillus subtilis* and *Bacillus licheniformis* and incubated at 37°C for 24 hours. One milliliter of each dilution and pH was then added to petridishes prefilled with 9 mL of PCA, incubated at same condition and ultimately colony forming unit (CFU) enumerated as CFU/mL for each dilution. According to the national standard for probiotics, (ISIRI, 2012) the cfu/mL should not be less than 10<sup>6</sup>.

#### *Resistance to artificial gastric juice (pepsin and trypsin)*

These tests required simulated gastric-fluid media containing pepsin, trypsin and sodium chloride at pH 2.3 and 7 (as control). Two hundreds µL of the bacterial suspensions (separately) were then removed, inoculated to each prepared artificial stomach media and incubated at 37°C for 2, 4 and 24 hours using the method of Jeon *et al.* (2017) with minor modification. Afterwards, 1 mL from each incubated media was added to tubes prefilled with 9 mL peptone solution and serial dilutions were continuously performed up to 10<sup>-8</sup> of which, 100 µl were inoculated in to petridishes prefilled with 10 mL PCA media and incubated at 37°C for 2, 4

and 24 hours. The cfu/mL was then counted. It should be noted that the number of colonies should not be less than

$10^6$  CFU/g (Shabani, 2008; ISIRI, 2012). Eventually, 24 treatments for the simulating stomach juice were prepared (Table 2).

**Table 2: The factorial design contained variables, bacteria (1= *B. subtilis*, 2= *B. licheniformis*), Stomach juice (1=pepsin, 2=trypsin), Time (1= 2 h, 2= 4 h and 3= 24 h) and pH (1= 2.3 and 2=7).**

Species of bacteria	S. Juice	pH	Hours	Group NO.	Name of Groups	
<i>B. subtilis</i>	Pepsin	2.3	2	1	Bacteria1 *Stomach.J1 * pH1 *Time1	
		2.3	4	9	Bacteria1 *Stomach.J1 *pH1 *Time2	
		2.3	24	17	Bacteria1 *Stomach.J1 *pH1 *Time3	
		7	2	2	Bacteria1 *Stomach.J1 *pH2 *Time1	
		7	4	10	Bacteria1 *Stomach.J1 *pH2*Time2	
		7	24	18	Bacteria1 *Stomach.J1 *pH2*Time3	
		Trypsin	2.3	2	5	Bacteria1 *Stomach.J2*pH1*Time1
	2.3		4	13	Bacteria1 *Stomach.J2*pH1*Time2	
	2.3		24	21	Bacteria1 *Stomach.J2*pH1*Time3	
	7		2	6	Bacteria1 *Stomach.J2*pH2*Time1	
	7		4	14	Bacteria1 *Stomach.J2 *pH2 *Time2	
	7		24	22	Bacteria1 *Stomach.J2*pH2*Time3	
	<i>B. licheniformis</i>		Pepsin	2.3	2	3
		2.3		4	11	Bacteria2 *Stomach.J1 *pH1 *Time2
2.3		24		19	Bacteria2*Stomach.J1*pH1*Time3	
7		2		4	Bacteria2 *Stomach.J1 *pH2*Time1	
7		4		12	Bacteria2 *Stomach.J1 *pH2 *Time2	
7		24		20	Bacteria2 *Stomach.J1 *pH2*Time3	
Trypsin		2.3		2	7	Bacteria2 *Stomach.J2 *pH1 *Time1
		2.3	4	15	Bacteria2 *Stomach.J2 *PH1 *Time2	
		2.3	24	23	Bacteria2*Stomach.J2*pH1*Time3	
		7	2	8	Bacteria2 *Stomach.J2 *pH2*Time1	
		7	4	16	Bacteria2 *Stomach.J2 *PH2*Time2	
		7	24	24	Bacteria2*Stomach.J2*pH2*Time3	

#### *Test of resistance to bile salts*

Tow hundreds  $\mu\text{L}$  of bacterial suspensions were equally inoculated into 2 tubes. One tube prefilled with 10 mL of BHI (Brain Heart Infusion) containing bile oxalate at concentrations of 0.3% and another had no bile salts. They were then incubated at  $37^\circ\text{C}$  for 8 h (Seifzadeh *et al.*, 2019). Ultimately, the absorbance (OD) of the bacterial suspension was measured at

600 nm before and after incubation. The rate of inhibitory was calculated as follows:

$$\text{Cinh} = \frac{(\text{T8}-\text{T0})\text{Control}-(\text{T8}-\text{T0})\text{Treatment}}{(\text{T8}-\text{T0})\text{Control}}$$

Where,  $\Delta\text{T8} - \text{T0}$  represents the difference in absorbance at time zero ( $\text{T0}$ ) and after 8 hours ( $\text{T8}$ ). Cinh of less than 0.4 was considered significant for



the isolates to be considered a suitable probiotic candidate.

#### *Hemolytic activity*

A loopfull of each isolated bacterium was linearly cultured on a blood agar with 5% defibrinated sheep blood (pH=7.2) and incubated under aerobic conditions overnight at 37°C (Banerjee *et al.*, 2017).

#### *Oxidase and Catalase reactions*

Some bacteria, such as *Bacillus*, which have the catalase enzyme, can form air bubbles after adding a speck of the bacterial colony to a 5% H<sub>2</sub>O<sub>2</sub> drop, indicating molecular oxygen being escaped (Sharifuzzaman *et al.*, 2018). A Whatman filter paper, which was triplicate folded was moistened with 1% aqueous solutions of tetramethyl-p-phenylenediamine dihydrochloride (Sigma-Aldrich), attaching with a bacterial speck of a single colony which was grown overnight on TSA involved to the moistened paper and showed a discoloration to deep purple (Sharifuzzaman *et al.*, 2018; Yi *et al.*, 2019).

#### *Antimicrobial activity*

One of the important pathogens of rainbow trout, *A. hydrophila* (ATCC 7966), as lyophilized bacteria was provided from the Faculty of Veterinary Medicine, University of Tehran to evaluate the antimicrobial activity of the isolated *Bacillus*. The Rimler-Shotts medium was used to

cultivate the pathogenic bacteria under the aerobic conditions.

#### *Well diffusion method*

Pathogenic *A. hydrophila* was linearly cultivated on blood agar plates. Four wells were then made in the plates for different microbial suspensions (200 µL) as 4 groups (1-4) at concentrations of control, 10<sup>4</sup>, 10<sup>6</sup>, and 10<sup>8</sup> CFU/mL, which control was incorporated with sterile physiological serum. They were then added to the wells and stored at 37°C for 24 hours.

#### *Disk diffusion method*

Twelve disks were equally incorporated with 180 µL (15 µL each) of microbial suspensions at concentrations of control, 10<sup>4</sup>, 10<sup>6</sup>, and 10<sup>8</sup> cell/mL in triplicate as 4 groups (1-4) so that 15 µL of physiological serum was added to the control disk. The incorporated disks were then placed in the middle of the plates prefilled with the blood agar and pathogenic bacteria (one concentration per plate). Finally, all the plates were incubated under the aerobic conditions at 37°C for 24 h.

#### *Data analysis*

The SPSS version 23 was used for the statistical analysis. Independent two-sample *t*-test was applied to examine the resistance of *B. subtilis* and *B. licheniformis* to gastric acid and bile salts. The general linear model and one-way ANOVA repeated measures were used to analyze the data from the gastric juice resistance test (pepsin and

trypsin). Moreover, one-way ANOVA test was used to analyze the microbial properties on well and disk diffusion tests. All the tests were carried out in triplicate.

## Results

### *Characterization of the bacteria*

The results of Gram reaction besides the chemical tests indicated that the predominant bacteria were *Bacillus* sp. According to Table 1, both isolated bacteria were Gram-positive, catalase-positive, and oxidase-negative.

Table 3 shows morphological, physiological and biochemical of *B. subtilis* Ahari.H1 and *B. licheniformis* Ahari.H2 with accession number in Genbank. The identification of the bacterial strain was carried out respect to non-molecular tests as well as 16S rDNA sequence analysis. Figure 1 shows Microscopic characteristics of the two bacteria. It was showed that the enumeration of the isolated bacteria, *B. subtilis* and *B. licheniformis* were  $3.2 \times 10^7$  CFU/g and  $1.2 \times 10^6$  CFU/g, respectively.

Microscopic characteristics (Table 3) of both predominant bacteria indicated that the colonies of the two isolates were aerobic, large-opaque adherent ones with irregular edges, of which *B. subtilis* were small rod chain Gram-positive and spore forming bacteria with varying cell size and *B. licheniformis* were large-rod chain Gram positive with sporulation characteristic (Fig. 1). Major physicochemical attribute of these two

isolates and their cultivation features on different carbohydrates and chemical media showed both bacteria deployed xylose to produce acid (doubtful for *B. subtilis*) and was unable to produce acid from galactose (doubtful for *B. subtilis*).

### *Hemolytic activity*

Interestingly, the results of the hemolysis tests for both bacteria, *B. subtilis* and *B. licheniformis* were negative and showed no hemolytic activity.

### *Gel electrophoresis of PCR products*

Fig. 2 Gel-Doc system (Fermentas; marker size, 100-3000 bp; band length, 2000 bp) demonstrates the electrophoresis of 16S rRNA gene sequence products for the isolated from *O. mykiss*. As shown (Fig. 2), replicated sequences (1500 bp) were observed in lanes 4, 5, 8, 9, 11, and 12, indicated that both microorganisms were *B. subtilis* and *B. licheniformis* as described in Figure 2.

### *The PCR assay*

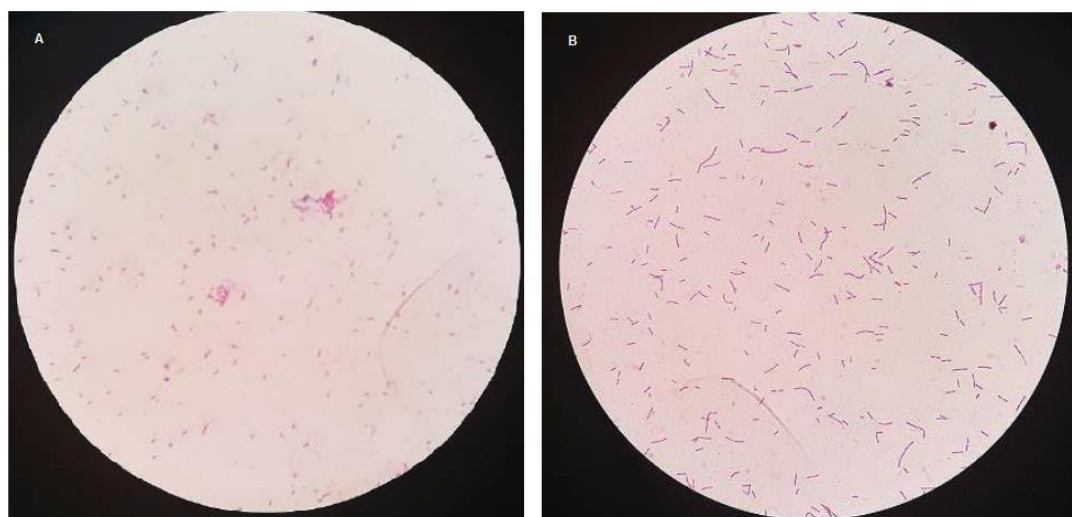
#### *Molecular characterization*

The partial sequences of the 16s rRNA gene obtained for both isolates were compared with the ones available in the NCBI database (December 2019) using BLASTn. Study of the strains showed 100% similarity to *Bacillus subtilis* strain XJ06 and less than 98% similarity to *B. licheniformis* strain SP8 and were named as *B. subtilis* Ahari.H1 and *B. licheniformis* Ahari.H2, respectively.

**Table 3: Characteristics of *Bacillus subtilis* and *Bacillus licheniformis***

parameters	Characteristics of isolated Bacillus	
	<i>Bacillus subtilis</i>	<i>Bacillus licheniformis</i>
GenBank Accession	MN809400	MN809401
Colony Morphology	Large opaque irregular edges	Large opaque irregular edges
Count (CFU/g)	$3.2 \times 10^7$	$1.2 \times 10^6$
Cell Morphology	Rods with rounded ends	Rods with rounded ends
Gram Reaction	+	+
Motility	+	+
VP	+	d
Spore Formation	+	+
Catalase	+	+
Urease	-	d
Oxidase	-	-
Galactose	d	-
xylose	d	+

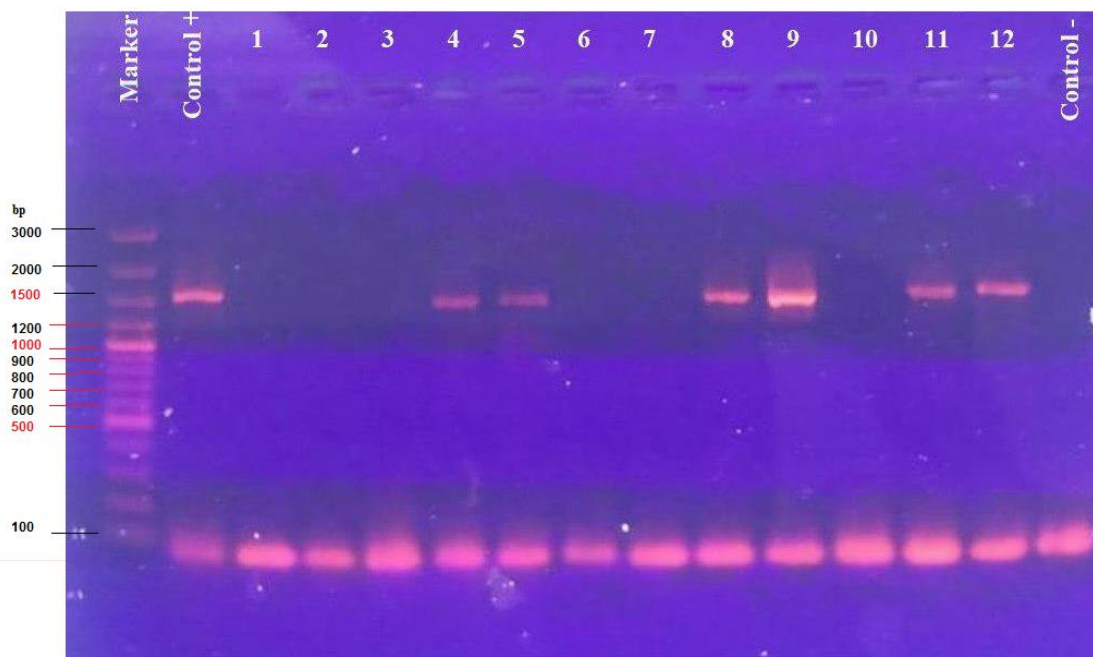
d= doubtful

**Figure 1: Microscopic characteristics of *B. licheniformis* (A) and *B. licheniformis* (B).**

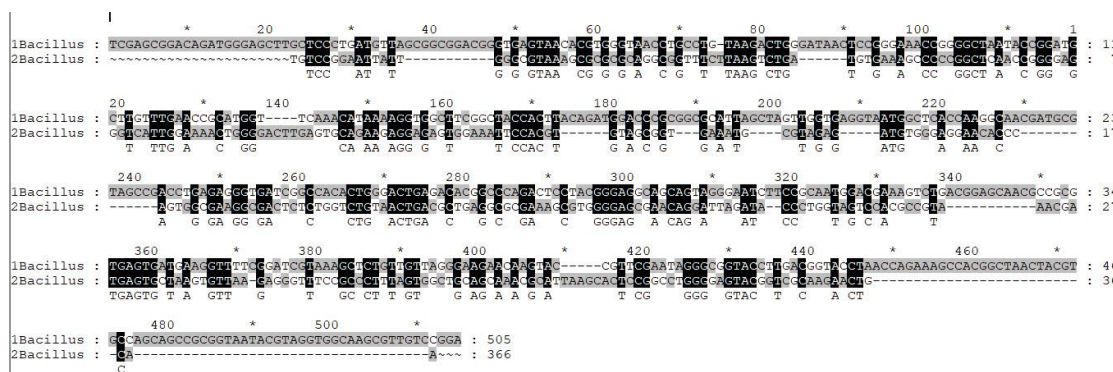
Alignment of the most highly conserved domain of the 16S rRNA gene using Gendoc and DNAMAN analysis are shown as figures 1 and 2. Conserved residues are highlighted in black. The percentage sequence similarity of two studied strains was 28.12% (Figs. 3 and 4). Based on figure 4, the percent of identity was 28.12% that was calculated based on the two sequences as they appeared in the multiple alignments.

#### *Resistance of the bacteria to acid and bile salts*

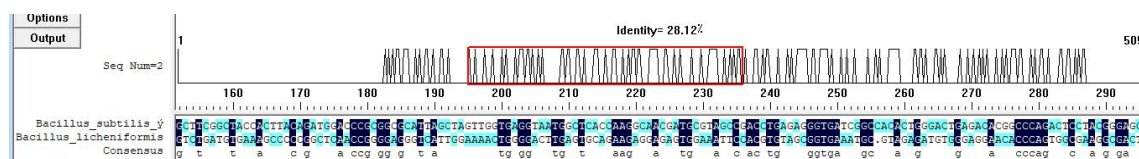
The number of bacteria grown under acidic conditions (pH 2.5 and 4) was greater than the standard level ( $10^6$  CFU  $g^{-1}$ ) so that after 24 hours of incubation, the mentioned bacteria were significantly grown in acidic media at pH 2.5 and 4. Furthermore, the number of bacterial colonies was almost similar to both pH.



**Figure 2:** Electrophoresis of the amplification products of 16S rRNA gene sequences for *Bacillus* isolates. From the left, first column was marker with the different bp, second one was the positive control with 1538 bp, lanes 5, 9 and 12 were dedicated to *B. subtilis* and lanes of 4, 8 and 11 were related to *B. licheniformis*. Lanes including 1-3, 6, 7 and 10 were empty and lane 13 was control. Moreover, the positive control contained *B. subtilis* strain B-37, which has been registered in the GenBank (No. KT948079.1). The negative control contained distilled water.



**Figure 3:** Evaluation of differences in the sequences of *Bacillus subtilis* and *Bacillus licheniformis* strains by GenDoc analysis. Identical conserved nucleotides are indicated by black highlights.



**Figure 4:** Evaluation of differences in the sequences of *Bacillus subtilis* and *Bacillus licheniformis* strains by DNAMAN analysis. Conserved residues are highlighted in black

Based on the results of independent *t*-test for *B. subtilis* and *B. licheniformis*, it was found that the mean number of growing colonies at pH of 2.5 (respectively,  $180 \pm 37.1 \times 10^7$  CFU g<sup>-1</sup> and  $160 \pm 24.0 \times 10^7$  CFU g<sup>-1</sup>) was significantly different from the mean number of growing colonies at pH of 4 (respectively,  $200 \pm 31.7 \times 10^7$  CFU g<sup>-1</sup> and  $201 \pm 60.0 \times 10^7$  CFU g<sup>-1</sup>) in both bacteria ( $p < 0.05$ ).

Both species were significantly resistant to bile oxalate at concentrations of 0.3%. The results showed that the rate of inhibition for *B. subtilis* and *B. licheniformis* were 0.142 and 0.285, respectively.

#### *Resistance to gastric juice (pepsin and trypsin)*

A number of grown colonies for both bacteria were measured for each plate. Therefore, the detected bacteria showed no susceptibility to gastric juice at 2, 4, and even at 24 h post-incubation (Fig. 5). The mean value of bacteria (log CFU g<sup>-1</sup>) grown under controlled condition (pH 7) as well as pH 2.3 exceeded  $10^8$  CFU g<sup>-1</sup>. Regardless the bacteria subjected to pH 7 on which bacteria grown more greater, there was no significant difference in the number of colonies (log CFU g<sup>-1</sup>) for both bacteria, *B. subtilis* and *B. licheniformis* at pH 2.3 ( $p > 0.05$ ) so that the lowest log CFU g<sup>-1</sup> of colony showed in group 3 on which *B. licheniformis* subjected to pepsin after 2 h ( $8.25 \pm 0.15$ ) but the greatest log CFU g<sup>-1</sup> represented in

groups 13 and 15 on which respectively *B. subtilis* ( $9.07 \pm 0.04$ ) and *B. licheniformis* ( $9.07 \pm 0.04$ ) subjected to trypsin after 4 h incubation. According to Figure 5, the number of log CFU/g of colonies for both bacteria exposed to pepsin, varied at different incubation periods and pH ranges (2.3 and 7).

#### *Antimicrobial activity test results*

##### *Well diffusion test*

On the well diffusion test, the highest level of antimicrobial activity of both bacteria was related to the dilution  $10^8$  CFU ml<sup>-1</sup> from bacterial suspensions. According to the results of ANOVA test for both bacteria ( $p < 0.01$ ), there was a significant difference between the mean of the four groups ( $p < 0.05$ ; Table 4). Bonferroni's test for *B. subtilis* confirmed the results presented in Table 4, and a significant difference was found ( $p < 0.05$ ). Moreover, the results for *B. licheniformis* confirmed the data presented in Table 4. According to this table, there was a significant difference between the groups ( $p < 0.05$ ), while for groups 2 and 3 ( $p = 1.00$ ), there was no significant difference ( $p > 0.05$ ).

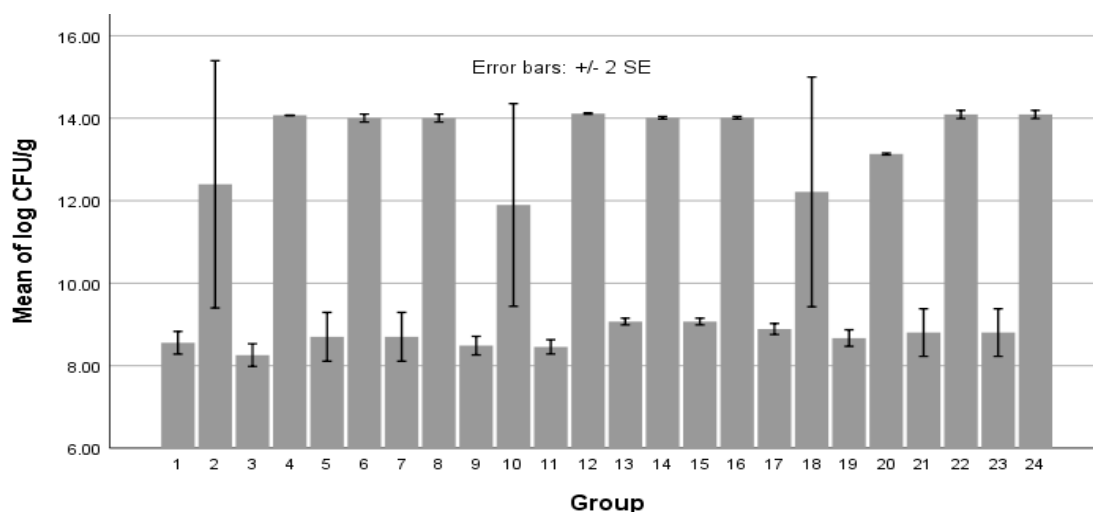


Figure 5: The effect of simulating stomach juice on growing *B. subtilis* and *B. licheniformis* (log CFU g<sup>-1</sup>).

Odd and even numbers are respectively related to pH 2.3 and 7.0 (control). The factorial test contained few variables such as bacteria (1= *B. subtilis*, 2= *B. licheniformis*), Stomach juice (1=pepsin, 2=trypsin), Time (1= 2 h, 2= 4 h and 3= 24 h), pH (1= 2.3 and 2=7). The grouping values were defined as follows: 1=bacteria1\*Stomach.J1\* pH1\*Time1, 2= Bacteria1 \*Stomach.J1 \*pH2 \*Time1, 3= Bacteria2 \*Stomach.J1\*pH1\*Time1, 4= Bacteria2 \*Stomach.J1 \*pH2\*Time1, 5= Bacteria1\*Stomach.J2\*pH1\*Time1, 6= Bacteria1\*Stomach.J2\*pH2\*Time1, 7= Bacteria2 \*Stomach.J2 \*pH1 \*Time1, 8= Bacteria2 \*Stomach.J2 \*pH2\*Time1, 9= Bacteria1 \*Stomach.J1 \*pH1\*Time2, 10= Bacteria1\*Stomach.J1 \*pH2\*Time2, 11= Bacteria2 \*Stomach.J1 \*pH1 \*Time2, 12= Bacteria2 \*Stomach.J1 \*pH2 \*Time2, 13= Bacteria1\*Stomach.J2\*pH1\*Time2, 14= Bacteria1 \*Stomach.J2 \*pH2 \*Time2, 15= Bacteria2 \*Stomach.J2 \*pH1\*Time2, 16=Bacteria2 \*Stomach.J2 \*pH2\*Time2, 17= Bacteria1 \*Stomach.J1 \*pH1 \*Time3, 18= Bacteria1\*Stomach.J1\*pH2\*Time3, 19= Bacteria2\*Stomach.J1\*pH1\*Time3, 20= Bacteria2 \*Stomach.J1 \*pH2\*Time3, 21= Bacteria1\*Stomach.J2\*pH1\*Time3, 22=Bacteria1\*Stomach.J2\*pH2\*Time3, 23=Bacteria2\* Stomach.J2\* pH1\*Time3, 24=Bacteria2\*Stomach.J2\*pH2\*Time3

Table 4: The mean diameter of the growth inhibition zone in the well diffusion method for *B. subtilis*

Groups	Concentration (Cell ml <sup>-1</sup> )	inhibition zone (mm)
<i>B. subtilis</i>	0	0 ± 0 <sup>a</sup>
	10 <sup>4</sup>	12 ± 0.75 <sup>b</sup>
	10 <sup>6</sup>	14±0.43 <sup>c</sup>
	10 <sup>8</sup>	17±0.28 <sup>d</sup>
<i>B. licheniformis</i>	0	0 ± 0 <sup>a</sup>
	10 <sup>4</sup>	11 ± 0.75 <sup>b,e</sup>
	10 <sup>6</sup>	12 ± 0.72 <sup>c,e</sup>
	10 <sup>8</sup>	15 ± 0.69 <sup>d</sup>

Dissimilar letters indicate significant differences between the groups ( $p < 0.05$ ).

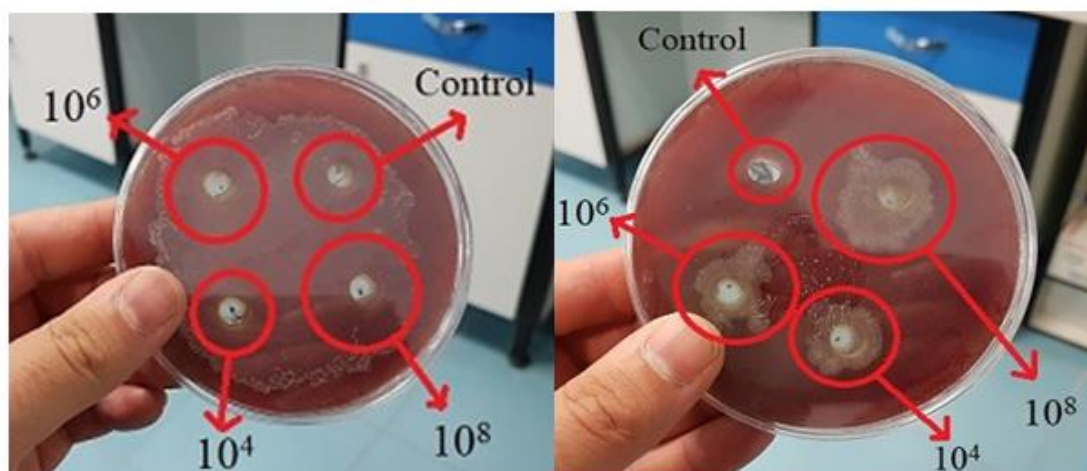
#### Disk diffusion test

According to the results of the well diffusion test, the highest antimicrobial activity of both bacteria in the disk

diffusion method was related to the dilution of 10<sup>8</sup> CFU ml<sup>-1</sup> from the bacterial suspension (Fig. 6). The one-way ANOVA test was used to analyze

the results of the disk diffusion test. The findings showed a  $P$ -value of 0.000 on the ANOVA test for both bacteria ( $p < 0.05$ ). Therefore, there was a significant difference for both bacteria between the groups ( $p < 0.05$ ). It should be noted that after the Bonferroni test, a significant difference was found between the 4 groups ( $p < 0.05$ ), while

there was no significant difference between groups 2 and 3 or groups 3 and 4 for both bacteria. No significant difference was observed between groups 2 and 4 for *B. subtilis* ( $p > 0.05$ ). Therefore, it can be concluded that in both well and disk diffusion tests, *B. subtilis* and *B. licheniformis* could inhibit the pathogenic *A. hydrophila*.



**Figure 6:** The growth inhibition zone of *B. subtilis* (Left) and *B. licheniformis* (Right). The greatest inhibitory activity was reported at a concentration of  $10^8$  CFU ml $^{-1}$  for *B. subtilis* and *B. licheniformis*, and the lowest inhibitory activity for *B. subtilis* was reported at a concentration of  $10^4$  CFU ml $^{-1}$ ; the control well contained sterile physiological serum without any growth inhibition.

As demonstrated in Table 4, the greatest mean diameter of the growth inhibition zone was found at a concentration of  $10^8$  CFU ml $^{-1}$  for *B. subtilis* and the lowest mean diameter was observed at a concentration of  $10^4$  CFU ml $^{-1}$ ; the growth inhibition zone diameter of the control was 0 mm. As shown in Table 4, the highest mean diameter of the growth inhibition zone of *B. licheniformis* was seen at the concentration of  $10^8$  CFU ml $^{-1}$ , and the lowest mean diameter was related to the control (0 mm). Moreover,  $10^6$  and  $10^4$

concentrations had almost similar growth inhibition zones, without any significant difference ( $p > 0.05$ ).

### Discussion

Extensive research has been conducted to investigate the bacterial flora of rainbow trout, indicating the presence of probiotic bacteria in the intestine. In the present study, the molecular detection of bacteria was performed based on 16S rRNA gene. According to accession number and the results (Table 3), the detected bacteria were *B. subtilis*

and *B. licheniformis*, which they were the dominant flora in the intestine of the rainbow trout. In another study (Ramesh *et al.*, 2015) *B. licheniformis* and *B. pumilus* were shown to be dominant flora in the intestines of *Labeo rohita*. Contrary to the results of this study, Didinen *et al.* (2018) isolated and detected *L. Lactis* subsp. *lactis* and *L. sakei* from rainbow trout, using biochemical and molecular methods based on 16S rRNA gene.

The values of *B. subtilis* MSM and *B. licheniformis* MR 78 were respectively  $1 \times 10^7$  and  $1 \times 10^8$  CFU ml<sup>-1</sup> after 4 hours of incubation (Seifzadeh *et al.*, 2019). Similarly, These results showed that *B. subtilis* isolated from digestive tract in midgut-intestinal canal was a prevalent bacteria with the highest value ( $3.2 \times 10^7$  CFU g<sup>-1</sup>) greater than another research (Merrifield *et al.*, 2010) showed the levels of *Bacillus* spp. was  $3.2 \times 10^6$  CFU ml<sup>-1</sup> while it showed  $0.74 \times 10^6$  CFU ml<sup>-1</sup> in the supplied water. Nevertheless, both values were greater than the standard emphasized on the minimum level of the probiotic (CFU g<sup>-1</sup>) isolated from intestine should not be more than  $1 \times 10^6$  (ISIRI, 2012). The great amplification of the bacteria observed in these results could be due to more activity of proteolysis function and high nutrients in anterior and mid-intestine tract compared with posterior tract (Enferadi *et al.*, 2018). Out of principals for approval a bacterium as a probiotic, the lack of hemolytic activity is the crucial attribute (Taheur *et al.*, 2016). Based on

our results, no hemolysis was observed due to the activity of the isolated strains.

Sorroza *et al.* (2012) showed the *V. fluvialis* (dominant flora in the intestines) was not resistant to pH below 5, similar to the results reported by Karim *et al.* (2016) regarding *Lactobacillus* isolated from the intestines of *Sperata seenghala* and *L. bata* at pH 2-7 showed that the growth of *Lactobacillus* isolated from the intestines was nil at pH 2 and maximum at pH 7 (neutral). The results of this study were contrary to finding of (Ramesh *et al.*, 2015) which showed *B. licheniformis* and *B. pumilus* extracted from the intestines of *L. rohita* bacteria had a relatively high resistance to low pH, 1, 2, and 3. The values of *B. subtilis* and *B. licheniformis* were,  $180 \pm 37.1 \times 10^7$  CFU g<sup>-1</sup> and  $160 \pm 24.0 \times 10^7$  CFU g<sup>-1</sup> respectively. It shows the resistance to pH at 2.3, which was greater than standard (ISIRI, 2012) but in agreement with our statement. This result also confirmed that both bacteria were significantly resistant to bile oxalate at concentrations of 0.3%, which was in line with the findings of Ramesh *et al.* (2015) that showed *B. licheniformis* and *B. pumilus* isolated from the intestines of *Labeo rohita* could survive at high concentrations, 2.5%, 5%, 7.5%, and 10% of bile salts. The study by Maji *et al.* (2016) on *L. plantarum* isolated from the intestines of rohu, catla, mrigal, silver carp, and grass carp confirmed that it could tolerates 0.3% bile salt for 24 hours.



The lowest rate of growth and activity of probiotic, *L. mesenteroides* was reported at pH 2, against pH 7, which showed the highest growth and activity of the probiotic. Similar to our results, the probiotic survived and multiplied at both concentrations of bile 0.15% and 0.3% as well as antimicrobial potential against pathogenic *A. hydrophila*, *Pseudomonas aeruginosa*, and *Shewanella putrefaciens*. (Allameh *et al.*, 2012). Similarly, *L. plantarum* VSG3 shown inhibitory effects against pathogenic *A. hydrophila* on *L. rohita* (Sankar *et al.*, 2013). Numerous strains of bacillus represent surprising biological appearances such as spore-forming feature enabling them to quickly proliferate and resist to destructive environmental conditions as well as different situations of acid and bile of digestive tracts of fish and shrimps (Soltani *et al.*, 2019). When *B. subtilis* was being cultured at unusual condition for 48 h in a culture medium, the sporulation efficiency was 28.44% (Zhang *et al.*, 2016). This could partially respond to the question revealed in this study that how a bacteria, could resist to gastric pH properly and can be extensively multiplied and found dominantly in the gut of trout. Both values for bacteria showed log CFU g<sup>-1</sup> exceeded than 8 (Fig. 5) under different conditions of gastric juice (pepsin and trypsin) remarkably more than standard (ISIRI, 2012). The *B. licheniformis* subjected to pepsin showed the lowest log CFU/g

(8.25±0.15) after 2 h of incubation but the greatest log CFU g<sup>-1</sup> represented for *B. subtilis* (9.07±0.04) and *B. licheniformis* (9.07±0.04) subjected to trypsin after 4 h incubation. These findings indicated that *B. subtilis* and *B. licheniformis* were resistant to gastric juice, which were not in line with the results of Maji *et al.* (2016) that showed *L. plantarum*, *L. pentosus*, *L. fermentum*, isolated from the intestines of some fishes could not tolerate the condition of gastric juice (pepsin and trypsin).

Based on the antimicrobial activity using well and disk diffusion tests, *B. subtilis* and *B. licheniformis* could inhibit the growth of virulent *A. hydrophila*. Mean diameter of growth inhibition zone was 17±0.28 mm while *B. subtilis* value was 1×10<sup>8</sup> cell/mL dissimilar to that of *B. licheniformis* that was measured 15 ± 0.69 mm at the same concentration (Table 4). These findings are in agreement with the results of Sorroza *et al.* (2012). They explained that *V. fluvialis* isolated from the intestines of fishes could prevent the growth of pathogenic *V. anguillarum* using well diffusion test. Similarly, Allameh *et al.* (2012) showed *L. mesenteroides*, isolated from snakehead fish showed inhibitory activities against pathogenic *A. hydrophila*, *Sh. putrefaciens*, and *Pseudomonas aeruginosa* on disk and well diffusion tests.

The detected bacteria were named as *B. subtilis* Ahari H.1 and *B. licheniformis* Ahari H.2, which they

were dominant flora in the intestine of the rainbow trout. In the current study, the isolated bacteria, *Bacillus subtilis* Ahari H.1 and *B. licheniformis* Ahari H.2 showed several attributes in *O. mykiss* containing tolerance to acidic pH (2.5 and 4), gastric juice (pepsin and trypsin) as well as to bile salts at concentrations of 0.3%. It is concluded that the detected bacteria could inhibit the growth of pathogenic bacteria, *A. hydrophila*.

### Acknowledgments

The authors express their sincere appreciation to the Islamic Azad University (IAU) for support of the student thesis.

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